



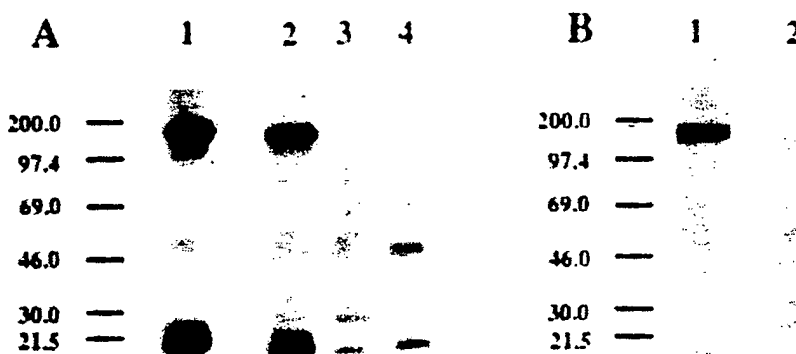
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(21) International Application Number: PCT/GB92/01506		(74) Agent: STUART, Ian; Mewburn Ellis, 2 Cursitor Street, London EC4A 1BQ (GB).	
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(71) Applicant (for all designated States except US): MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB).		Published With international search report.	
(72) Inventors; and (75) Inventors/Applicants (for US only) : BRENNAN, Mary [GB/GB]; PUKLAVEC, Michael, Joseph [GB/GB]; MRC Cellular Immunology Unit, Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE (GB).			

(54) Title: MONOCLONAL ANTIBODY SPECIFIC FOR AN ANTIGEN ON DENDRITIC CELLS



(57) Abstract

Ligands are provided which bind a binding partner antigen restricted to dendritic cells and a novel subpopulation of CD3+ cells. A hybridoma, MRC OX-62, which produces antibodies with this binding specificity has been deposited under the accession number ECACC 91061805. Techniques for obtaining further ligands, mutants, derivatives and functional equivalents are described, along with uses of these. The antibody immunoprecipitates dendritic cell antigens of approximately 150 KD. (major band), 120 KD (minor band) and two lower bands of < 30 KD apparent Mr.

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MONOCLONAL ANTIBODY SPECIFIC FOR AN ANTIGEN ON DENDRITIC CELLS

The present invention relates to ligands for dendritic cells. In particular, the present invention relates to ligands for dendritic cells which are antibodies especially monoclonal antibodies (mAbs), and to derivatives, fragments and functional equivalents of these antibodies.

Lymphoid dendritic cells are a heterogeneous group of cells with dendritic morphology found in lymphoid and non-lymphoid tissues (reviewed in 1). Dendritic cells originate from bone marrow, but the lineage of dendritic cells is unresolved (reviewed in 2). Attention has been focused on dendritic cells following the observation that these cells are the most potent accessory cells identified at inducing primary T-cell responses in vitro (3). The immunoregulatory role of dendritic cells in vivo is implicated by their presence in the T-dependent areas of secondary lymphoid organs permitting T-cell activation (4,5) and in the thymic medulla (6).

Identification of dendritic cells is based on morphology (8), constitutive expression of MHC class II (9), low phagocytic ability in vitro (10) and potent accessory function in the primary allogenic mixed leucocyte reaction (MLR) (3). The need for mAbs that are specific for dendritic cells is evident (reviewed in 11), but few useful mAbs are available (12-18) and anti-

MHC class II mAbs are of limited value. One major problem in generating dendritic cell mAbs, is that it is difficult to obtain large numbers of dendritic cells for immunization and there are no cell lines equivalent to normal dendritic cells (19-21).

The present application discloses a methodology for making ligands e.g. mAbs which are highly specific for dendritic cells and which also bind a subpopulation of CD3⁺ cells, and provides a mAb produced by carrying out the disclosed methodology. The antibody and cell line producing this antibody are both designated MRC OX-62.

The MRC OX-62 cell line secretes a mouse IgG1 mAb, highly restricted to rat dendritic cells and a subpopulation of CD3⁺ cells as determined by immunocytochemistry, flow cytofluorography and in vitro proliferative responses. The MRC OX-62 mAb immunoprecipitates four bands from radioiodinated dendritic cells under both non-reducing and reducing conditions: a major band of approximately 150 kD apparent Mr, a minor band of approximately 120 kD apparent Mr, and two lower bands <30 kD apparent Mr. The 150 kD apparent Mr band is the only band detected by the MRC OX-62 mAb on Western blots.

In lymphoid organs, the MRC OX-62 mAb predominantly labels populations of cells with dendritic morphology in the thymic medulla, T-cell areas and red pulp of the spleen, the subcapsular sinus, T-cell areas and medulla of the lymph node, inter-follicular areas and epithelial

regions of the Peyer's patch and veiled cells in lymph. In non-lymphoid organs, the MRC OX-62 mAb labels populations mostly coinciding with known dendritic cell distribution. Enrichment of dendritic cells assessed by
5 potent stimulator activity in the primary allogenic MLR can be effectively achieved by magnetic sorting after labelling cells with the MRC OX-62 mAb.

Therefore, the present invention provides the hybridoma MRC OX-62, mAbs produced by said hybridoma and
10 derivatives, functional equivalents and fragments of these antibodies.

The hybridoma MRC OX-62 has been deposited under the terms of the Budapest Treaty at ECACC on 18 June 1991 under accession No. 91061805.

15 It will be understood by those skilled in the art that the hybridoma may be subject to genetic mutation or other changes, while still retaining its ability to produce monoclonal antibody of the same specificity. The present invention therefore encompasses mutants,
20 other derivatives and descendants of the hybridoma MRC OX-62.

It will be further understood by those skilled in the art that a monoclonal antibody can be subjected to the techniques of recombinant DNA technology to produce
25 derivative other antibodies or chimeric molecules which retain the specificity of the original monoclonal antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the

complementarity determining regions (CDR's), of the monoclonal antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin, for example to convert the mouse-derived
5 monoclonal antibody into one having largely human immunoglobulin characteristics (see EP 184187A, GB 2188638A).

EP-A-O 120 694 (Boss et al/Celltech) describes the cloning and expression of chimeric antibodies. In these
10 derivatives the variable domains from one immunoglobulin are fused to constant domains from another immunoglobulin. Usually, the variable domains are derived from an immunoglobulin from one species, say a mouse or a rat, and the constant domains are derived
15 from an immunoglobulin from a different species, perhaps a human. This technology is now very well known in the art. A later European Patent Application, EP-A-O 125 023 (Cabilly/Genentech), also US 4816567, describes much the same subject as the Boss patent application, but
20 describes production of other variations of immunoglobulin-type molecules using recombinant DNA technology.

Another possibility is to attach just the variable region of the monoclonal antibody to another non-
25 immunoglobulin molecule, to produce a derivative chimeric molecule (see WO 86/01533, Neuberger and Rabbits/Celltech). A further possibility would be to produce a chimeric immunoglobulin having different

specificity in its different variable regions, one of which is that the monoclonal antibody of the present invention (see EP 68763A). Yet another possibility would be to produce a mutation in the DNA encoding the monoclonal antibody, so as to alter certain of its characteristics without changing its essential specificity. This can be done by site-directed mutagenesis or other techniques known in the art.

The famous Winter patent application EP-A-O 239 400 describes how it is possible to make an altered, derivative, antibody by replacing the complementarity determining regions (CDRs) of a variable region of an immunoglobulin with the CDRs from an immunoglobulin of different specificity, using recombinant DNA techniques, so called "CDR-grafting". This enables antigens-binding specificity of one antibody (in the present case it might be MRC OX-62 mAb or an antibody with the same binding specificity or an antibody which is cross-reactive with MRC OX-62 mAb) to be transferred to another antibody. This enables "humanisation" of antibodies. A "humanised" antibody with the CDRs of a rat antibody specific for an antigen of interest, might well be less likely to be recognised as foreign by the immune system of a human. It follows that a "humanised" antibody with the same binding specificity as MRC OX-62 mAb or cross-reactive with it (see later), might well be of particular use in human therapy and/or diagnostic methods. The uses of an antibody according to the

present invention are discussed later in the text. All such uses of the monoclonal antibody are encompassed by the present invention.

As discussed, the state of the art is such that the person skilled in the art well knows how to manipulate and alter any given antibody to form a derivative to suit his or her particular needs.

The provision of the MRC OX-62 mAb allows persons skilled in the art to obtain binding partners e.g. antigens or epitopes which bind to it. These partners may be rat dendritic cells originally utilised as the antigen, the novel subpopulation of CD3⁺ cells identified using MRC OX-62 mAb, or homologue cell populations from other species, or parts e.g. epitopes of said cells. The ability to obtain these binding partners by use of MRC OX-62 mAb will overcome the prior art problem relating to the difficulty in obtaining large numbers of dendritic cells for immunization. Therefore, the present invention also provides binding partners e.g. antigens and/or epitopes which bind with said MRC OX-62 mAb. In particular, binding partners obtained by contacting a sample suspected of containing a binding partner with MRC OX-62 mAb, are provided. The binding partner may comprise part or all of a 150 kD protein derived from dendritic cells.

The binding partners obtained by use of the MRC OX-62 mAb may also be used to produce further ligands e.g. antibodies (or molecules having antibody-like binding

function e.g. fragments, derivatives and synthetic analogues of antibodies) other than the MRC OX-62 mAb. Therefore, also provided are ligands e.g. mAbs which are able to bind with a binding partner which is able to bind with the MRC OX-62 mAb. Such ligands ("cross-reactive ligands") e.g. mAbs may recognize different epitopes, or the same epitope as recognized by MRC OX-62 mAb on said binding partner.

The present invention also provides derivatives, functional equivalents (e.g. a molecule having an antibody-like binding specificity) and fragments of said cross-reactive ligands, perhaps produced using one or more of the techniques of recombinant DNA technology referred to and discussed above. Also included are single domain ligands (dAbs) as described in WO 90/05144 (Winter et al/MRC).

Using standard techniques, it is possible to use the antibody of the present invention in immunopurification of a binding partner antigen. Techniques for immunoaffinity column purification are well known, see for instance "Current Protocols in Immunology", ed. J E Coligan et al, John Wiley and Sons, Unit 8.2. In fact, it should be possible to use an immunoaffinity column to isolate cross-reactive ligands as discussed above, without needing to isolate the antigen itself. A first round of immunoaffinity uses the MRC OX-62 mAb to take out from a sample the antigen binding partner, which may then be used in the column to

select from a heterogeneous population of ligands, those ligands which are cross-reactive with the MRC OX-62 mAb, ie which bind the same binding partner.

A binding partner isolated using the MRC OX-62 mAb
5 maybe used to select cross-reactive ligands from a repertoire or heterogeneous population of antibodies generated by a whole variety of means. One way is to select monoclonal antibodies and cell lines producing them by the standard hybridoma techniques. Also
10 provided by the present invention are immortalised cells e.g. hybridomas producing said cross-reactive ligands.

The present invention also provides processes for the preparation of immortalised cell lines (e.g. hybridomas), and ligands produced by these immortalised
15 cell lines which comprise immunising a mammal with an antigenic binding partner which binds with said MRC OX-62 mAb; immortalising antibody-producing cells from said mammal; selecting immortalised cells producing a ligand which binds with said binding partner; and cloning said
20 selected cells.

The antibody-producing cells may be spleen cells.

The immortalisation step may be carried out by fusing said spleen cells with myeloma cells according to techniques known in the art.

25 Another way of selecting ligands which are cross-reactive with the MRC OX-62 mAb is to use the methods for producing members of specific binding pairs disclosed in WO 92/01047 (Cambridge Antibody Technology

Limited and MRC/McCafferty et al). This discloses expression of polypeptide chain components of a genetically diverse population of specific binding pair members, such as antibodies, fused to a component of a secreted replicable genetic display package (RGDP), such as a bacteriophage, which thereby displays the polypeptide on the surface. Very large repertoires of displayed antibodies may be generated, and screened by means of antigen binding to obtain one or more antibodies of interest, along with their encoding DNA. DNA encoding for a polypeptide displayed on the surface of an RGDP is contained within that RDGP and may therefore be easily isolated and cloned for expression. The antibody repertoire screen may of course be derived from a human source.

Obviously, once one has an immortalised cell line, e.g. a hybridoma, or an RGDP containing DNA encoding at least a polypeptide component of a binding ligand, one skilled in the art is in a position to obtain (according to techniques well known in the art) the nucleotide sequence encoding the ligand e.g. the mAb secreted by the cell. Therefore, the present invention also encompasses primary nucleotide sequences which encode the ligands e.g. mAbs as defined above, together with fragments of these primary sequences and secondary nucleotide sequences comprising derivatives, mutations and hybridising partners of said primary nucleotide sequences.

These nucleotide sequences may be used in a recombinant systems to produce an expression product according to standard techniques. Therefore, the present invention includes vectors (cloning and expression vectors) incorporating said nucleotide sequences, transformed cells incorporating said vectors and expression products produced by use of a recombinant system utilising any such vectors or transformed cells.

The present invention also includes methods for expressing a ligand e.g. a mAb, derivative, functional equivalent or fragment thereof, which comprises using a nucleotide sequence, vector or transformed cell as defined above.

More specifically, MRC OX-62 mAb will bind to an epitope on rat dendritic cells. This epitope may then be purified, for instance utilising an immunoaffinity column (as discussed), and partially or wholly sequenced, for instance using repeated rounds of Edman degradation. An analysis of the sequence allows the production of a nucleotide sequence (e.g. the construction of a synthetic nucleotide sequence) which encodes at least part or all of the epitope. The nucleotide sequence can then be used as a probe to screen rat dendritic cells for hybridising mRNA species. Conversion of the hybridising mRNA into cDNA provides a rat cDNA probe which can be used to identify human homologues of the rat binding partner for MRC OX-62. Two routes are possible and other routes will be

apparent to those skilled in the art:

(i) using the rat cDNA as a probe, to screen human cDNA or genomic libraries at low stringency; and

(ii) using the rat sequence for the design of
5 oligonucleotides to use as primers in the PCR (polymerase chain reaction). PCR is very well known in the art, but one may refer to US patent 4683 195 (Mullis et al) or Saiki et al, Science 230, pp 1350-1354, 1985, for a general description of the technique. Primers
10 will be in both the "sense" and "antisense" orientations and as many pairs as necessary may be tested in the PCR. As a starting point oligonucleotides are made to sequences likely to be conserved between the rat and human homologues. cDNA can be synthesized by standard
15 procedures from sources likely to contain the human homologue e.g. human dendritic cells. The conditions for the polymerase chain receptor (PCR) might involve trials at a number of low initial temperatures to accommodate any mismatches (Gould, S.J., Subramani, S.
20 and Scheffler, I.E. (1989) Proc. Natl. Acad. Sci. USA 86, 1934-1938) but otherwise under standard conditions. The PCR products can then be analysed by sizing on agarose or other gels, transfer to nitrocellulose or similar membranes and probed with the rat cDNA under
25 non-stringent conditions for cross species hybridization. PCR products corresponding to human homologue will be sequenced by standard methods. (These products could also be used as probes as in (1) for

screening cDNA or genomic libraries).

The availability of a cDNA for the human homologue allows the testing of various human cell populations for expression of the human homologue either by PCR or by Northern blot analysis. Production of a human homologue using for example prokaryotic or eukaryotic expression systems will provide material for the production of ligands e.g. polyclonal or monoclonal antibodies or other proteins with an epitope-binding function.

The MRC OX-62 mAb, derivatives, fragments and functional equivalents may provide means for manipulating antigen presentation in the tissues. Coupling a peptide onto dendritic cells by means of the antibody may permit more effective presentation of that peptide as antigen for T-cell mediated immunity. Such an adjuvant effect would be useful in vaccination strategies.

The mAbs, derivatives, functional equivalents and fragments thereof as defined above have a number of useful applications based upon their specificity to dendritic cells and a novel subpopulation of CD3⁺ cells.

Ligands as provided above and especially ligands for human homologues of the rat binding partner for MRC OX-62 can be used for the pretreatment of tissue and organ grafts and for the management of graft rejection. Previous work has shown restoration of immunogenicity to passenger cell depleted kidney allografts by the addition of donor strain dendritic cells (Lechler, R.I.

and Batchelor, J.R. 1982 J. Exp. Med. 155:31).

Thus the present invention encompasses a preparation for the treatment of foreign tissue which comprises a ligand e.g. an antibody as defined above or
5 a fragment, derivative or functional equivalent thereof in a perfusion medium.

The foreign tissue may be any tissue which would be seen by a recipient as not originating from said recipient. Typically, the foreign tissue may be
10 xenogeneic or allogenic.

In autoimmune diseases, diabetes and cancers there is frequently an accumulation of dendritic cells in the affected tissues. Therefore, the present invention also includes pharmaceutical preparations for the
15 inactivation and/or depletion of dendritic cells from cancers or tissues adversely affected by autoimmune disease, which comprise a ligand e.g. an antibody, or a fragment, derivative or functional equivalent thereof, as herein provided, together with one or more
20 excipients.

The ligands e.g. antibodies or fragments, derivatives or functional equivalents thereof may also be used to diagnose the accumulation of dendritic cells which is indicative of an abnormal disease state.

25 Therefore, the present invention also includes a diagnostic reagent which comprises a ligand e.g. an antibody, or a fragment, derivative or functional equivalent thereof, as herein provided, together with

one or more exipients; a diagnostic kit comprising a diagnostic reagent and a diagnostic method which comprises contacting a ligand e.g. antibody, fragment, derivative or functional equivalent thereof as herein
5 provided with a clinical tissue sample and detecting the binding of said ligand to said sample.

The ligands as provided above and/or binding partners for these ligands can be used for the targeting of antigens. Therefore the present invention
10 encompasses an antigen targeting method which comprises using a ligand and/or binding partner as herein provided to target an antigen. The present invention also provides materials comprising a ligand and/or binding partner as herein provided for use in such a method.

15 In order that the present invention is more clearly understood, embodiments of the invention will be described in more detail with reference to the figures as described below.

Some abbreviations used herein mean the following:

20 MLNX - mesenteric lymphadenectomized rats;

TDL - thoracic duct leukocytes

LPS - Lipopolysaccharide;

RAM - rabbit anti-mouse

FITC - fluorescein isothiocyanate

25 PMSF - phenyl methyl sulphonyl fluoride

Figure 1

Binding of OX-62 mAb to PVG rat lymphoid tissues.
Cryostat sections (5µm) of PVG thymus, spleen, cervical

lymph node and Peyer's patch labelled using the indirect immunoperoxidase method with OX-62 mAb and isotype-matched negative control OX-21 mAb. A. Thymus labelled with OX-62 mAb. Cells in the medulla (M), scattered
5 cells in the cortex (C) and infrequent cells extending into the lobular septae (arrow head) labelled. B. Thymus labelled with OX-21 mAb. C. Spleen labelled with OX-62 mAb. Cells in the T-cell areas (T) of the white pulp surrounding the central arterioles (arrow heads),
10 infrequent cells in the B-cell areas (B) of the white pulp and cells in the red pulp (RP) labelled. D. Spleen labelled with OX-21 mAb. Low levels of non-specific labelling. E. Cervical lymph node labelled with OX-62 mAb. Cells in the sub-capsular sinus (arrow heads) and
15 paracortex (P) labelled but few cells in the follicle (F) labelled. F. Cervical lymph node labelled with OX-21 mAb. G. Peyer's patch labelled with OX-62 mAb. Cells in the inter-follicular T-dependent areas (T) and dome epithelium (arrow) labelled but few cells in the
20 follicle (F) labelled. H. Peyer's patch labelled with OX-21 mAb. I. PVG MLNX TDL labelled with OX-62 mAb. Note labelling of cells with dendritic morphology (arrow heads). J. PVG TDL labelled with OX-62 mAb. Magnification: A-H x60, I and J, x375.

25 Figure 2

Cryostat sections (5µm) of PVG lamina propria (A and B) and epidermal PVG ear sheets (C-G). A-D labelled using the indirect immunoperoxidase method. E-F

labelled using double immunofluorescence. (A) OX-62 mAb. Cells in the villus with dendritic morphology and smaller intra-epithelial cells (arrow) labelled. (B) OX-6 mAb. Cells in the villus with cytoplasmic processes and some cells extending into the epithelium (arrow heads) labelled. Note granular OX-6 mAb labelling of the epithelium (B. arrow) compared to the OX-62 mAb intra-epithelial cell labelling (A. arrow). Magnification x250. (C) OX-62 mAb. Note labelling of dendritic processes. (D) OX-6 mAb. Note rounded morphology and less prominent dendrites. Heavy non-specific labelling of the hair follicles (arrow heads). Magnification x125. (E) GAM-FITC detection of OX-62 mAb. (F) streptavidin Texas red detection of OX-6 IgG biotin-conjugated mAb. (G) combined GAM-FITC detection of the OX-62 mAb and streptavidin Texas red detection of OX-6 IgG biotin-conjugated mAb. Note non-overlapping populations of OX-62⁺ and OX-6⁺ cells. Examples of nonoverlapping OX-62⁺ cells (arrow) in (E) and (G) are missing in (F) Examples of OX-6⁺ cells (arrow head) in (F) and (G) are missing in (E) Large fluorescent areas represent autofluorescence of hair follicles (E-G).

Figure 3

Binding of the OX-62 mAb to different isolated PVG cell populations. Cells were labelled with saturating levels of OX-62 mAb (-) and OX-21 mAb (--) followed by a second incubation with RAM-FITC. Bound mAb was measured by flow cytometry. (A) TDL, (B) MLNX TDL, (C) MLNX TDL

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gradient enriched for dendritic cells (50% enrichment),
(D) PBL, (E) bone marrow cells, (F) thymocytes, (G)
splenocytes, (H) lymph node cells, (I) resident
peritoneal exudate cells, (J) thioglycollate elicited
5 peritoneal exudate cells, (K) LPS blasts, (L) Con A
blasts. Note that all profiles are superimposed except
for C and D.

Figure 4

Comparison of magnetically sorted OX-62⁺ enriched
10 and depleted populations as stimulators in an MLR. 5×10^5 AO TDL responders depleted of OX-6, OX-17, OX-8 and
OX-12 labelled cells by magnetic sorting were stimulated
with different numbers of unseparated PVG MLNX TDL (●),
OX-62 magnetically enriched PVG MLNX TDL (■), and OX-62
15 magnetically depleted PVG MLNX TDL (□). The data shown
are the mean and standard deviation of quadruplicate
cultures. The graph shows counts $\times 10^4$ v cell number.

Figure 5

(A) Immunoprecipitation of the OX-62 antigen from a
20 ¹²⁵I-labelled dendritic cell lysate. Samples were run
on 7.5% SDS-PAGE prior to autoradiography. Numbers on
the left indicates the apparent Mr (kD) determined from
market proteins. Samples were unreduced (lane 1) or

reduced (lanes 2-4). (1) and (2) OX-62 immunoprecipitation following control SN3 mAb preclear. (3) OX-62 immunoprecipitation following OX-62 preclear. (4) Control SN3 mAb immunoprecipitation after control SN3 mAb preclear.

(B) Western blotting of the OX-62 antigen. Dendritic cell lysates were run on 7.5% SDS-PAGE and electroblotted to nitrocellulose. The nitrocellulose membranes were probed with (lane 1) OX-62 mAb, (lane 2) isotype-matched negative control OX-21 mAb, and ^{125}I -RAM as the second mAb prior to autoradiography. Numbering on the left indicates the apparent Mr (kD) determined from marker proteins.

MATERIALS AND METHODS

Animals

Mice: Balb/c (H-2^d) and DBA/2 (H-2^d) inbred mice were obtained from the Sir William Dunn School of Pathology, Oxford. F1 hybrids between these two strains were bred at the MRC Cellular Immunology Unit, Oxford.

Rats: PVG (RT1^C) and AO (RT1^U) specific pathogen-free inbred rats were obtained from the MRC Cellular Immunology Unit, Oxford.

Suitable animals may be obtained from standard suppliers.

Mesenteric Lymphadenectomy

Caecal, Mesenteric, post-gastric and portal nodes were surgically removed using blunt dissection from 90g

male PVG rats. Mesenteric lymphadenectomized (MLNX) rats were allowed to recover for a minimum of 6 wks before thoracic duct cannulation.

Cell Populations

5 Thoracic duct leukocytes (TDL) were obtained by thoracic duct cannulation of normal and MLNX rats (22). Cells were collected overnight into ice cold glucose saline (23) containing 20U/ml heparin. Density gradient enriched dendritic cells were prepared by centrifugation
10 of MLNX TDL over NycoprepTM 1.068 (Nycomed, Oslo, Norway). Splenocytes, lymph node cells and thymocytes were obtained by removing appropriate organs and teasing into single cell suspensions in PBS/0.25% BSA. PBL were prepared from blood obtained by cardiac puncture and
15 separated over Isopaque-Ficoll. Bone marrow cells were obtained by flushing the marrow cavity of the femur with PBS. Resident peritoneal exudate cells were obtained from the peritoneal cavity of freshly killed rats. Elicited peritoneal exudate cells were obtained from the
20 peritoneal cavity 4 d after i.p. injection of 10 mls of thioglycollate broth. Con A blasts were obtained following 3 d culture of lymph node cells at 10^6 /ml in RPMI 1640 supplemented with 5% heat inactivated FCS, 2mM glutamine, 1mM sodium pyruvate, 2.5×10^{-5} M 2-
25 mercaptoethanol and antibiotics (supplemented RPMI) and 5µg/ml Con A. Con A blasts were separated over Isopaque-Ficoll and washed in α -methyl-D-mannoside (20 mg/ml). LPS blasts were obtained following 2 d culture

of lymph node cells at 10^6 /ml in supplemented RPMI 1640 and 10 μ g/ml LPS.

Monoclonal antibodies

- 5 OX-62 was produced by immunizing a Balb/c mouse with rat PVG density gradient enriched dendritic cells obtained from the cannulated thoracic duct of MLNX rats. The immunization procedure comprised of a combination of i.v. and i.p. injections with $5-10 \times 10^6$ cells at
- 10 monthly intervals. 5 d after the last injection the splenocytes were fused with NSO myeloma cells according to the method of Galfrè & Milstein (24). After growth of the hybrid cell lines supernatants were screened for specificity by immunocytochemistry (see below).
- 15 Selected hybridomas were cloned twice by limiting dilution. Ascites fluid was prepared in (Balb/c X DBA/2)F1 mice pretreated with pristane. The subclass of the mAbs were determined by an anti-mouse monoclonal isotyping kit (Serotec, Kidlington, Oxon. England.).
- 20 Other mAbs used were SN3 (mouse anti-squid Sgp 1) (25), OX-6 (mouse anti-rat MHC class II: IgG1) (26), OX-8 (mouse anti-rat CD8: IgG1) (27), OX-12 (mouse anti-rat IgG kappa chain: IgG2a) (28), OX-17 (mouse anti-rat MHC class II: IgG1) (29). OX-21 (mouse anti-human C3b
- 25 inactivator: IgG1) (3); OX-30 (mouse anti-rat CD45: IgG2a) (31) and OX-42 (mouse anti-rat CD18/CD11b: IgG2a) (32). MAbs were used as tissue culture supernatants and purified IgG from ascites fluid (33).

Other antibodies used were rabbit anti-mouse IgG mAb peroxidase conjugated (RAM-peroxidase) (Dako, High Wycombe, Bucks. England.), and affinity purified rabbit F(ab')₂ anti-mouse IgG mAb (Serotec, Kidlington, Oxon. England.) conjugated with fluorescein (RAM-FITC) (34),
5 biotin (RAM-biotin) (34) and iodine (¹²⁵I-RAM) (35).

Preparation of specimens for immunocytochemistry

Cryostat sections: Thymuses, spleens, cervical lymph nodes and Peyer's patches were removed from 8-12
10 wk PVG rats embedded in Tissue-TekTM OCT (Miles, Elkhart, Ind., USA) and frozen in 2-methylbutane cooled in liquid nitrogen. 5 µm cryostat sections were cut into 4-spot glass slides (C.A. Hendley, Essex, England.) air dried and stored desiccated at -70°C.

15 Epidermal sheets: PVG rat ears were split longitudinally and incubated for 2-4 h at 37°C in a 5% CO₂ incubator in 116 mM NaCl, 2.6 mM KCl, 8 mM Na₂HPO₄ and 20 mM Na₄EDTA pH 7.3 (36). Epidermal sheets were separated using watchmaker's forceps under a dissecting
20 microscope.

Isolated cells: Cytospin preparations were made onto glass slides using a cytocentrifuge, air dried and stored dessicated at -70°C.

25 Immunocytochemistry

Staining was carried out using the peroxidase method as described previously (37). Briefly, cryostat sections, epidermal sheets and isolated cell

preparations were fixed in ethanol, washed and incubated with mAb tissue culture supernatant, washed and incubated with RAM-peroxidase. Peroxidase was visualized using 3,3'-diaminobenzidine tetrahydrochloride. Slides were lightly counterstained with Harris' haematoxylin, dehydrated and mounted in DPX. Epidermal sheets were stained in wells and incubated with mAb tissue culture supernatants overnight and with RAM-peroxidase for 90 min.

10 Flow cytofluorography

Labelling of cells for analysis on a FACScan (Becton Dickinson, Mountain View, California, USA) was performed as described previously (34). Cells were gated on the scatter profiles to exclude dead cells and erythrocytes.

15 Identification of phagocytic cells

25 µl colloidal carbon (Pelikan ink) was injected i.v. and 24 h later organs were removed and frozen for cryostat sections for immunocytochemistry.

20 Magnetic cell sorting

The principle of the magnetic cell sorting system and instructions for use have been described in detail (41,42). Briefly, cells were labelled with mAb tissue culture supernatants ($50 \mu\text{l}/10^7$) for 1 h at 40°C , washed, labelled with RAM-biotin ($50 \mu\text{l}$ of $10 \mu\text{g}/\text{ml}/10^7$ cells) for 30 min at 40°C , washed, labelled with avidin-FITC (Becton-Dickinson, Cowley, Oxford, England.) ($50 \mu\text{l}$ of $20 \mu\text{g}/\text{ml}/10^7$ cells) for 30 min at 40°C washed and

finally incubated with biotinylated superparamagnetic microbeads (Becton-Dickinson, Cowley, Oxford, England.) (5 μ l/ 10^8 cells/ml) for 5 min at 40°C. The magnetic separation was carried out at 40°C. Labelled cells were
5 added to the appropriately sized column which had been preincubated with PBS/0.25%BSA/10 mM NaN₃ washed and placed in the magnetic field. Unlabelled cells were collected in the first 10-15 ml volume. The column was washed to remove unlabelled, weakly bound and dead
10 cells. Magnetically labelled cells were eluted from the column outside the magnetic field using a 50 ml syringe filled with PBS/0.25%BSA/10 mM NaN₃. In some experiments enriched and depleted populations were further depleted of contaminating cells by a second
15 magnetic sort. The efficiency of depletion and enrichment was determined by flow cytofluorography using a FACScan.

MLR

5 x 10^5 responder cells were co-cultured with
20 different numbers of irradiated (20 Gy) stimulator cells in supplemented RPMI in 96-well round bottomed micro-tire plates for 72 h at 37°C in a 5% CO₂ incubator. In some experiments OX-62 and appropriate control MAbs were added as purified IgG at the start of culture. Samples
25 were pulsed with 0.5 μ Ci tritiated thymidine [³H-TdR] for a further 18 h. Samples were then harvested for incorporation of ³H-TdR.

Cell surface radioiodination and immunoprecipitation

10^7 density gradient enriched cells were surface labelled by an H_2O_2 lactoperoxidase-catalysed radioiodination method (38). Labelled cells were solubilized in 500 μ l lysis buffer comprising 10 mM tris
5 pH 7.5-8 150 mM NaCl, 5 mM Na_2EDTA , 1% Nonidet P-40, 1mM PMSF and 5 mM iodoacetamide for 30 min at 40°C. Samples were centrifuged and the supernatants precleared for 30 min at 40°C on a rotating wheel with 100 μ l of a 10% suspension of SepharoseTM CL-4B beads (Pharmacia,
10 Uppsala, Sweden) which had been covalently coupled to purified mAb IgG using cyanogen bromide (approximately 10 mg purified mAb IgG/ml CL-4B beads) (39). Preclearing was repeated three times. Precleared samples were directly immunoprecipitated for 1 h at 40°C
15 on a rotating wheel with 30 μ l of a 10% solution of SepharoseTM CL-4B beads covalently coupled to purified mAb IgG. Immunoprecipitated samples were washed in the following buffers (1) 10 mM tris-HCl pH 8.0 containing 500 mM NaCl, 0.5% NP-40 and 0.05% SDS (b) 10 mM tris-HCl
20 pH 8.0 containing 150 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate and 0.05% SDS (c) 10 mM tris-HCl pH 8.0 containing 0.05% SDS. Immunoprecipitates were released from the beads by boiling for 4 min in SDS-PAGE sample buffer. Samples and molecular weight markers (RainbowTM
25 protein molecular weight markers. Amersham, Bucks, England.) were analysed by SDS-PAGE under non-reducing and reducing conditions on 7.5% resolving acrylamide gels using a Mini-Protean 11 dual slab cell (Bio-Rad)

(40). Gels were fixed, dried and exposed to HyperfilmTM (Amersham, Bucks, England.) with two intensifying screens at -70°C.

Western blotting

5 Dendritic cell lysates prepared as described for immunoprecipitation were subjected to SDS-PAGE using a Mini-Protean 11 dual slab cell (Bio-Rad). Protein was transferred to nitrocellulose (HybondTM-C-extra-Amersham, Bucks, England.) using a mini trans-blot
10 electrophoretic transfer cell (Bio-Rad). Membranes were placed in PBS containing 5% dried non-fat milk powder (MarvelTM) for 24 h at 40°C. Ascites fluid containing the appropriate mAb was added to give a final concentration of 1-50 µg/ml and the membranes incubated
15 for 2 h at 40°C with rocking. The membranes were washed three times in PBS/0.05% TweenTM 20 before addition of ¹²⁵I-RAM (specific activity approximately 1.5 µCi/µg IgG at a dilution of 10⁶ cpm/ml) in PBS/0.05% Tween 20/1% BSA. After 1 h at 40°C the membranes were washed three
20 times in PBS/0.05% Tween 20 and exposed to HyperfilmTM at -70°C.

RESULTS AND DISCUSSION

Production of the OX-62 mAb

25 Out of a total of four fusions and screening approximately 2000 wells one hybridoma secreting a candidate mouse anti-rat dendritic cell IgG1 mAb (OX-62) was selected. The salient features contributing to the production of OX-62 were: 1. Cannulated MLNX rats were

used as a source of dendritic cells for injection. Isolating dendritic cells from lymph compared with lymphoid organs circumvents lengthy isolation procedures and possible modification of surface phenotypes (43).

- 5 2. Large numbers of dendritic cells ($5-10 \times 10^6$) were injected per mouse and each mouse received at least three injections. 3. Supernatants from the fusion were screened by immunocytochemistry using the indirect immunoperoxidase method on thymus, spleen, cervical lymph node and Peyer's patch sections. Binding assays are unlikely to detect dendritic cell specific mAbs unless enriched dendritic cells are used. In the current studies <10% of the mAbs produced were against MHC class II antigens as assessed by immunocytochemistry
- 10 although anti-MHC class II mAbs have been reported to comprise up to 50% of positive hybridomas in anti-dendritic cell fusions (18). A number of interesting mAbs against endothelial determinants were produced (data not shown).

20 Distribution of the OX-62 antigen in sections

The distribution of the OX-62 antigen in lymphoid organs (Table 1 and Fig. 1) mostly coincided with previous reports of dendritic cell distribution based on microscopic and immunocytochemical studies (thymus: 6, 44, 45, spleen: 4, 8, lymph node: 8, 46, Peyer's patch 8,

25 47).

All cells labelled with the OX-62 mAb had a dendritic cell morphology. In the thymus, OX-62⁺ cells

were present in the medulla forming a diffuse network (Fig. 1A). OX-62⁺ cells were also present in the lobular septae with occasional cells present in the cortex (Fig. 1A) correlating with the large scattered
5 cells observed in the cortex in bone marrow chimaeras (6). In the spleen OX-62⁺ cells were concentrated in the T-dependent areas particularly around the region of the central arteriole but also throughout the red pulp (Fig. 1C). Labelling of cells in the red pulp was
10 reported for NLDC 145 (13) and N418 (18) mAbs which recognize mouse dendritic cells. Rare OX-62⁺ cells were present in the marginal zones and the peripheral white pulp (Fig. 1C). The OX-62 mAb cells in the red pulp were more strongly stained compared with the T-dependent
15 areas of the spleen (Fig. 1C). Differences in expression may relate to morphology based on the observation that the OX-62⁺ cells in the red pulp appeared more rounded compared with cells in the T-dependent areas where the borders were difficult to
20 define (data not shown). In the cervical lymph node OX-62⁺ cells were present in the subcapsular sinus, T-dependent areas of the cortex and in the medulla but were absent from the follicles (Fig. 1E). In the Peyer's patch OX-62⁺ cells were present in the inter-
25 follicular areas and in the epithelial dome regions but were absent from the follicles (Fig. 1G). Background staining with an isotype matched mAb OX-21 was negligible (Fig. 1B, D, F and G). OX-62⁺ cells

represented a minor population of the OX-6⁺ and OX-42⁺ cells (data not shown).

Distribution of the OX-62 Antigen in Nonlymphoid Organs

OX-62⁺ cells with dendritic morphology that
5 coincided with known MHC class II dendritic cell
distribution (48, 63) were present in the lamina propria
of the small intestine (Fig. 2, A and B), interstitium
of the lung, portal triads of the liver, glomeruli of
the kidney, islets of Langerhans of the pancreas, and
10 epithelium of the choroid plexus (data not shown). Lack
of OX-62⁺ cells in heart and skeletal muscle (data not
shown) contrasts with previous studies using anti-MHC
class II mAbs (48, 63 and 64).

Unexpectedly, the OX-62 mAb revealed populations of
15 OX-62⁺ MHC class II⁻ cells in the epithelium of the
small intestine and in the epidermis of the skin. In
the epithelium of the small intestine, only granular MHC
class II staining has been reported (Fig. 2B; and 47),
but this staining was not associated with the OX-62⁺
20 intraepithelial cells (Fig. 2A). In epidermal sheets,
OX-62 mAb labelling primarily revealed cells with marked
dendritic morphology comprising the cell body and
nonoverlapping dendritic processes (Fig. 2C) in contrast
to OX-6 mAb labelling, which primarily revealed cells
25 with less prominent dendrites (Fig. 2D). Double
immunofluorescence showed that most if not all the OX-
62⁺ and MHC class II⁺ populations were nonoverlapping
(Fig. 2, E, F, and G). The OX-62⁺ MHC class II⁻ cells

in the small intestine and the skin were CD3⁺ (data not shown) and probably represent gamma/delta T cells (65-67). The identification of nonoverlapping OX-62⁺ MHC class II⁻ and OX-62⁻ MHC class II⁺ populations in the skin with a similar distribution raises the question as to which cells migrate into the afferent lymphatics giving rise to the OX-62⁺ MHC class II⁺ veiled cells. Previous work has indicated that MHC class II⁺ Langerhans cells (68) are the probably precursors (50, 69-71).

The presence of dendritic cells in autoimmune diseases has been documented (51 and reviewed in 52) therefore the distribution of OX-62⁺ cells in cryostat sections from rats with autoimmune diabetes and experimental allergic encephalomyelitis were examined. The OX-62 mAb labelled increased numbers of OX-62⁺ cells in the islets of Langerhans with leukocyte infiltrates in the pancreas of rats with autoimmune diabetes when compared to control non-diabetic rats (data not shown). Similarly increased OX-62 mAb labelling of cells in lesions in the spinal cord and brain of rats with experimental allergic encephalomyelitis was found in contrast to normal rats where OX-62⁺ cells are present in the choroid plexus (data not shown). It could be that the increased labelling represents upregulation of the OX-62 antigen or migration of OX-62⁺ cells into the lesions, but what has taken place has not been established.

Expression of the OX-62 antigen by different cell types

Fig. 3 shows FACScan profiles of different isolated types following labelling with the OX-62 mAb. Resident peritoneal macrophages (Fig. 3I), exudate peritoneal macrophages (Fig. 3J), LPS blasts (Fig. 3K) and Con A blasts (Fig. 3L) were OX-62⁺. Heterogeneous populations such as spleen, thymus and lymph node cells known to contain dendritic cells in low numbers by immunocytochemistry (Fig. 1A, C and E) gave staining with the OX-62 mAb at a level barely distinguishable from background (thymocytes Fig. 3F, splenocytes Fig. 3G and lymph node cells Fig. 3H). In contrast significant positive profiles following labelling with the OX-62 mAb were obtained with density gradient enriched MLNX TDL (Fig. 3C) indicating that the OX-62 antigen is expressed on the cell surface and that OX-62⁺ cells are normally present at too low a frequency to be clearly detectable using a FACScan. For this reason TDL and MLNX TDL were analysed by immunocytochemistry on slides. Veiled cells were the only cell type in MLNX TDL that labelled with the OX-62 mAb. There was a lack of labelling of TDL with the OX-62 mAb although very rare OX-62⁺ cells were observed (data not shown). Veiled cells are occasionally observed in efferent and central lymph.

Enrichment of dendritic cells

The infrequent distribution of dendritic cells makes isolation difficult. Dendritic cells can either be obtained in suspension in lymph or from lymphoid

organs with or without enzyme digestion and adherence. Dendritic cells represent approximately 1% or less of these populations therefore further enrichment is dependent either on negative selection using relevant
5 mAbs and/or non-specific separation using density gradients (reviewed in 60). Enrichment of dendritic cells by labelling cells with the N418 mAb followed by cell sorting using flow cytofluorography has been reported (61) but the low recoveries inherent in cell
10 sorting using flow cytofluorography (30% or less) combined with the low frequency of labelled cells in the presort population limits the number of dendritic cells obtainable. The ability of the OX-62 mAb to enrich and deplete for OX-62 labelled cells was assessed
15 phenotypically by flow cytofluorography using a FACScan after magnetic cell sorting and functionally using magnetically sorted cells as stimulators in the primary allogeneic MLR. The OX-62 mAb in combination with magnetic sorting gave 80% enrichment and recoveries were
20 approximately 90%. The potency of the cell populations separated after labelling with the OX-62 mAb and used as stimulators in the primary allogeneic MLR is shown in Fig. 4. The OX-62 enriched cells were potent stimulators with 1.22×10^3 cells per culture providing
25 the same stimulus as 15.5×10^3 unseparated cells. The depleted cells had 12% of the activity of the unseparated cells indicating that the OX-62⁺ cells were the major stimulating cell in the MLR. It can be argued

that almost all the stimulating activity is from OX-62⁺ cells since the activity of the depleted cells is in accord with the level of OX-62⁺ cell contamination. The OX-62 mAb in combination with magnetic sorting represents a useful method for enriching for dendritic cells in large numbers with greater than 80% purity more quickly than by cell sorting using flow cytometry. To obtain purer populations of dendritic cells magnetically sorted cells could be sorted by flow cytometry.

Effect of the OX-62 mAb on the primary allogeneic MLR

OX-62 IgG added at the start of culture in a concentration range from 1.25 ng/ml to 2.5 µg/ml had no effect on the MLR between CD4 responders from AO rats (i.e. OX-6, OX-17, OX-8 and OX-12 mAb labelled and magnetically depleted TDL) and unseparated PVG MLNX TDL stimulators (data not shown) indicating that the OX-62 antigen is not essential for T-cell activation. The anti-CD4 mAb W3/25 used as a positive control inhibited in the concentration range from 1.25 ng/ml to 2.5 µg/ml (data not shown).

Molecular characterization of OX-62 antigen

The OX-62 mAb immunoprecipitated four bands from radioiodinated dendritic cells under both non-reducing (Fig. 5A(1)) and reducing (Fig. 5A(2-4)) conditions: a major band of approximately 150 kD apparent Mr, a minor band of approximately 120 kD apparent Mr, and two lower bands <30 kD apparent Mr. The OX-62 mAb precleared all

bands immunoprecipitated by the OX-62 mAb but some contaminants remained in the lowest apparent Mr band, which coincided with the dye front (Fig. 5A(3)). OX-42 and OX-30 which are expressed on dendritic cells failed to preclear the bands immunoprecipitated by the OX-62 mAb (data not shown). The 150 kD apparent Mr band was the only band detected by the OX-62 mAb on Western blots (Fig. 5B).

The biochemical properties of the OX-62 binding partner antigen are characteristic of an integrin. Certain members of the integrin family under reducing conditions immunoprecipitate four bands representing the alpha and beta subunits (higher apparent Mr bands) and alternatively spliced cleavage products of the alpha subunit (lower apparent Mr bands) (reviewed in 60). The strong 150 kD band present under non-reducing and reducing conditions is alpha-subunit like and the weaker 120 kD band, which migrated faster under non-reducing conditions, is beta-subunit like. This 120 kD band could be present as a result of co-precipitation with the alpha-subunit (recognised by the OX-62 antibody), due to noncovalent association of the alpha and beta subunits. The presence of the two bands of less than 30 kD under non-reducing (fig. 5A (1)) and reducing (fig. 5A(2)) conditions, perhaps representing cleaved fragments, is not the same as previously described integrin alpha-subunits.

MRC OX-62 is a mAb highly restricted to dendritic

cells in lymphoid and non-lymphoid organs, and to a novel subpopulation of CD3+ cells with dendritic morphology.

5 The hybridoma MRC OX-62 has been deposited in accordance with the rules and regulations of the Budapest Treaty with the European Collection of Animal Cell Cultures (ECACC, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, UK) on 18 June 1991 under the
10 accession number 91061805.

REFERENCES

1. Metlay, J.P., Puré, and R.M. Steinman. 1989. Control of the immune response at the level of antigen-presenting cells: A comparison of the function of dendritic cells and B lymphocytes. *Adv. Immunol.* 47:45.
2. Fossum, S. 1989. The life history of dendritic leukocytes (DL). In *Current Topics in Pathology. Cell kinetics of the inflammatory reaction*. Vol. 79. (Ed. O.H. Iversen) Springer-Verlag, Berlin, Germany. Chapter V 101-124.
3. Steinman, R.M., and M.D. Witmer. 1978. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc. Natl. Acad. Sci.* 75:5132.
4. Veerman, A.J.P., and W. van Ewijk. 1975. White pulp compartments in the spleen of rats and mice. A light and electron microscopic study of lymphoid and non-lymphoid cell types in T- and B- areas. *Cell Tiss. Res.* 156:417.
5. Witmer, M.D., and R.M. Steinman. 1984. The anatomy of peripheral lymphoid organs with emphasis on accessory cells: Light-microscopic immunocytochemical studies of mouse spleen, lymph node, and Peyer's patch. *Am. J. Anat.* 170:465.
6. Barclay, A.N., and G. Mayrhofer. 1981. Bone marrow origin of Ia-positive cells in the medulla of rat thymus. *J. Exp. Med.* 153:1666.
7. Lo, D., R. Yacov, and J. Sprent. 1986. Induction of MHC-restricted specificity and tolerance in the thymus. *Immunol. Res.* 5:221.
8. Steinman, R.M. and Z.A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. 1. Morphology, quantitation, tissue distribution. *J. Exp. Med.* 137:1142.
9. Nussenzweig, M.C., R.M. Steinman, J.C. Unkeless, M.D. Witmer, B. Gutchinov, and Z.A. Cohn. 1981. Studies of the cell surface of mouse dendritic cells and other leukocytes. *J. Exp. Med.* 154:168.
10. Steinman, R.M., and Z.A. Cohn. 1974. Identification of a novel cell type in peripheral lymphoid organs of mice. 11. Functional properties in vitro. *J. Exp. Med.*

139:380.

11. MacPherson, G.G. 1989. Lymphoid dendritic cells: Their life history and roles in immune responses. *Res. Immunol.* 140:877.
12. Nussenzweig, M.C., R.M. Steinman, M.D. Witmer, and B. Gutchinov. 1982. A monoclonal antibody specific for mouse dendritic cells. *Proc. Natl. Acad. Sci.* 79:161.
13. Kraal, G., M. Breel, M. Janse, and G. Bruin. 1986. Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. *J. Exp. Med.* 163:981.
14. Poulter, L.W., D.A. Campbell, C. Munro, and G. Janossy. 1986. Discrimination of human macrophages and dendritic cells by means of monoclonal antibodies. *Scand. J. Immunol.* 24:351.
15. Breel, M., R.E. Mebius, and G. Kraal. 1987. Dendritic cells of the mouse recognized by two monoclonal antibodies. *Eur. J. Immunol.* 17:1555.
16. Nagelkerken, L.M., B. Schutte, R.J.M. Stet, and P.J.C. van Breda Vriesman. 1987. Recognition of rat dendritic cells by a monoclonal antibody. *Scand. J. Immunol.* 26:347.
17. Maruyama, T., S. Tanaka, B. Bozoky, F. Kobayashi, and H. Uda. 1989. New monoclonal antibody that specifically recognizes murine interdigitating and Langerhans cells. *Lab. Invest.* 61:98.
18. Metlay, J.P., M.D. Witmer-Pack, R. Agger, M.T. Crowley, D. Lawless, and R.M. Steinman. 1990. The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. *J. Exp. Med.* 171:1753.
19. Cohen, D.A., and A.M. Kaplan. 1981. Adherent Ia⁺ murine tumor lines with characteristics of dendritic cells. 1. Morphology, surface phenotype, and induction of syngeneic mixed lymphocyte reactions. *J. Exp. Med.* 154:1881.
20. Clayberger, C., R. DeKruyff, R. Fay, and H. Cantor. 1985. Identification of a unique B-cell-stimulating factor produced by a cloned dendritic cell. *Proc. Natl. Acad. Sci.* 82:183.
21. Breel, M., J. Laman, and G. Kraal. 1988. Murine hybrid cell lines expressing the NLDC-145

- dendritic cell determinant. *Immunobiol.* 178:167.
22. Ford, W.L., and S.V. Hunt. 1973. The preparation and labelling of lymphocytes. In *Handbook of Experimental Immunology* (Ed. D.M. Weir) Blackwell Scientific Publications, Oxford, England. Chapter 23, 23.1-23.27.
 23. de St. Groth, S.F., and D. Scheidegger. 1980. Production of monoclonal antibodies: strategy and tactics. *J. Immunol. Methods.* 35:1.
 24. Galfrè, G., and C. Milstein. 1981. Preparation of monoclonal antibodies: Strategies and procedures. *Methods in Enzymol.* 73:3.
 25. Williams, A.F., A.G.D. Tse, and J. Gagnon. 1988. Squid glycoproteins with structural similarities to Thy-1 and Ly-6 antigens. *Immunogenetics* 27:265.
 26. McMaster, W.R., and A.F. Williams. 1979. Identification of Ia glycoproteins in rat thymus and purification from rat spleen. *Eur. J. Immunol.* 9:426.
 27. Brideau, R.J., P.B. Carter, W.R. McMaster, D.W. Mason, and A.F. Williams. 1980. Two subsets of rat T lymphocytes defined with monoclonal antibodies. *Eur. J. Immunol.* 10:609.
 28. Hunt, S.V., and M.H. Fowler. 1981. A repopulation assay for B and T lymphocyte stem cells employing radiation chimaeras. *Cell Tissue Kinet.* 14:445.
 29. Fukumoto, T., W.R. McMaster, and A.F. Williams. 1982. Mouse monoclonal antibodies against rat major histocompatibility antigens. Two Ia antigens and expression of Ia and class I antigens in rat thymus. *Eur. J. Immunol.* 12:237.
 30. Hsiung, L., A.N. Barclay, M.R. Brandon, E. Sim, and R.R. Porter. 1982. Purification of human C3b inactivator by monoclonal-antibody affinity chromatography. *Biochem. J.* 203:293.
 31. Woollett, G.R., A.N. Barclay, M. Puklavec, and A.F. Williams. 1985. Molecular and antigenic heterogeneity of the rat leukocyte-common antigen from thymocytes and T and B lymphocytes. *Eur. J. Immunol.* 15:168.
 32. Robinson, A.P., T.M. White, and D.W. Mason. 1986. Macrophage heterogeneity in the rat as delineated by two monoclonal antibodies MRC OX-

- 41 and MRC OX-42, the latter recognizing complement receptor type 3. *Immunology* 57:239.
33. Mason, D.W., and A.F. Williams. 1980. The kinetics of antibody binding to membrane antigens in solution and at the cell surface. *Biochem. J.* 187:1.
34. Mason, D.W., W.J. Penhale, and J.D. Sedgwick. 1987. Preparation of lymphocyte subpopulations. In *Lymphocytes a practical approach* (Ed. G.G.B. Klaus) IRL Press, Oxford, England. Chapter 2. 35-54.
35. Jensenius J.C., and A.F. Williams. 1974. The binding of anti-immunoglobulin antibodies to rat thymocytes and the thoracic duct lymphocytes. *Eur. J. Immunol.* 4:91.
36. Bergstresser, P.R., and D.V. Juarez. 1984. Detection by immunochemical techniques of cell surface markers on epidermal Langerhans cells. *Methods in Enzymol.* 108:683.
37. Barclay, A.N. 1981. The localization of populations of lymphocytes defined by monoclonal antibodies in rat lymphoid tissues. *Immunology* 42:593.
38. Goding, J.W. 1986. Membrane and secretory immunoglobulins: structure, biosynthesis, and assembly. In *Handbook of Experimental Immunology* (Eds. D.M. Weir, L.A. Herzenberg, C. Blackwell, and Leonore A Herzenberg) Blackwell Scientific Publications, Oxford, England. Fourth Edition, Vol. 1, Chapter 20, 20.1-20.33.
39. Porath, J. 1974. General methods and coupling procedures. *Methods in Enzymol.* 73:13.
40. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680.
41. Sorting cells with MACS. Instructions for use. 1990. Miltenyi Biotec, Gladbach, West Germany.
42. Miltenyi, S., W. Müller, W. Weichel, and A. Radbruch. 1990. High gradient magnetic cell separation with MACS. *Cytometry* 11:231.
43. Girolmoni, G., J.C. Simon, P.R. Bergstresser, and P.D. Cruz, Jr. 1990. Freshly isolated spleen dendritic cells and epidermal Langerhans cells undergo similar phenotypic and functional changes during short term culture. *J. Immunol.*

145:2820.

44. Duijvestijn, A.M., and E.C.M. Hoefsmit. 1981. Ultrastructure of the rat thymus: the microenvironment of T-lymphocyte maturation. *Cell Tissue Res.* 218:279.
45. van Ewijk, W. 1984. Immunohistology of lymphoid and non-lymphoid cells in the thymus in relation to T lymphocyte differentiation. *Am. J. Anat.* 170:311.
46. Fossum, S. 1980. The architecture of rat lymph nodes. II. Lymph node compartments. *Scand. J. Immunol.* 12:411.
47. Mayrhofer, G., C.W. Pugh, and A.N. Barclay. 1983. The distribution, ontogeny and origin in the rat of Ia-positive cells with dendritic morphology and of Ia antigen in epithelia, with special reference to the intestine. *Eur. J. Immunol.* 13:112.
48. Steinman, R.M. 1991. The dendritic cell system and its role in immunogenicity. *Ann. Rev. Immunol.* 9:271.
49. Hart, D.N.J., and J.W. Fabre. 1981. Demonstration and characterization of Ia-positive dendritic cells in the interstitial connective tissues of rat heart and other tissues, but not brain. *J. Exp. Med.* 154:347.
50. Silberberg-Sinakin, I., G.J. Thorbecke, R.L. Baer, S.A. Rosenthal and V. Berezowsky. 1976. Antigen-bearing Langerhans cells in skin, dermal lymphatics and in lymph nodes. *Cell Immunol.* 25:137.
51. Knight, S.C., J. Martin, A. Stockpoole, and J. Clark. 1983. Induction of immune responses in vivo with small numbers of veiled (dendritic) cells. *Proc. Natl. Acad. Sci.* 80:6032.
52. King, P.D., and D.R. Katz. 1990. Mechanisms of dendritic cell function. *Immunol. Today* 11:206.
53. Liu, L.M., and G.G. MacPherson. 1990. Intestinal dendritic cells in the rat. Isolation and characterization. 10th International Conference on Lymphatic tissues and germinal centres in immune reactions, Compiègne, France. 7.12 (Abstr.).
54. Hopkins, J., B.M. Dutia, R. Bujdoso, and I. McConnell. 1989. In vivo modulation of CD1 and

- MHC class II expression by sheep afferent lymph dendritic cells. Comparison of primary and secondary immune responses. *J. Exp. Med.* 170:1303.
55. Inaba, K., M.D. Witmer-Pack, M. Inaba, S. Muramatsu, and R.M. Steinman. 1988. The function of Ia⁺ dendritic cells and Ia-dendritic cell precursors in thymocyte mitogenesis to lectin and lectin plus interleukin 1. *J. Exp. Med.* 167:149.
 56. Darden, A.G., R.D. Forbes, P.M. Darden, and R.D. Guttman. 1990. The effects of genetics and age on expression of MHC class II and CD4 antigens on rat cardiac interstitial dendritic cells. *Immunol.* 126:322.
 57. Guy-Grand, D., and P. Vassalli. 1988. Origin and traffic of gut mucosal lymphocytes and mast cells. In *Migration and homing of lymphoid cells.* (Ed. A.J. Husband) CRC Press, Inc., Boca Raton, FL. Vol. II, 99-111.
 58. Guy-Grand, D., N. Cerf-Bensussan, B. Malissen, M. Malassis-Seris, C. Briottet, and P. Vassalli. 1991. Two gut intraepithelial CD8⁺ lymphocyte populations with different T cell receptors: A role for the gut epithelium in T cell differentiation. *J. Exp. Med.* 173:471.
 59. Fangmann, J., R. Schwinzer, and K. Wonigeit. 1991. Unusual phenotype of intestinal intraepithelial lymphocytes in the rat: predominance of T cell receptor α/β^+ /CD2⁻ cells and high expression of RT6 alloantigen. *Eur. J. Immunol.* 21:753.
 60. Klinkert, W.E.F. 1990. Lymphoid dendritic accessory cells of the rat. *Immunol. Rev.* 117:103.
 61. Crowley, M.T., K. Inaba, M.D. Witmer-Pack, S. Gezelter, and R.M. Steinman. 1990. Use of the fluorescence activated cell sorter to enrich dendritic cells from mouse spleen. *J. Immunol. Methods* 133:55.
 62. Hemler, M.E. 1990. VLA proteins in the integrin family: structures, functions, and their role on leukocytes. *Ann. Rev. Immunol.* 8:365.
 63. Steiniger, B.J. Klempnauer, and K. Wonigeit. 1984. Phenotype and histological distribution of interstitial dendritic cells in the rat pancreas, liver, heart, and kidney. *Transplantation (Baltimore)* 38:169.

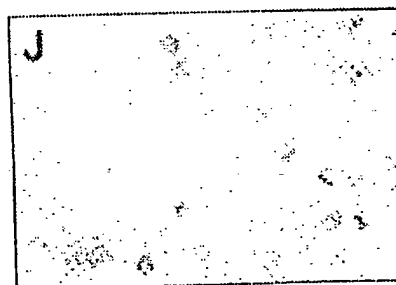
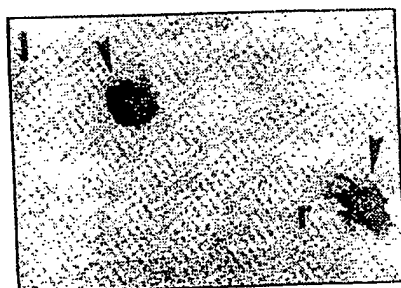
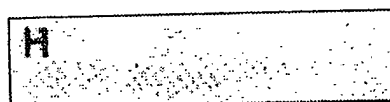
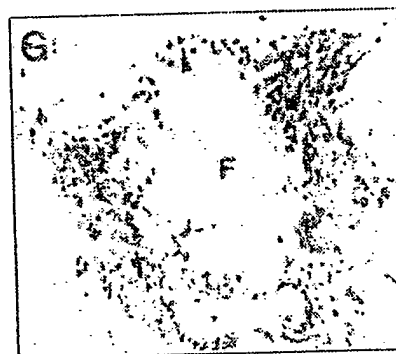
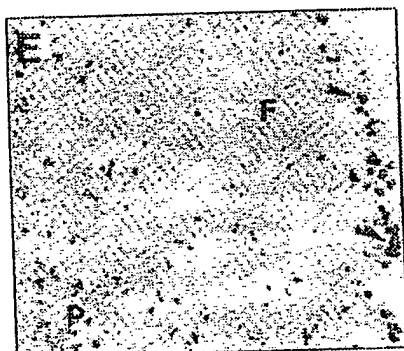
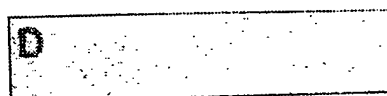
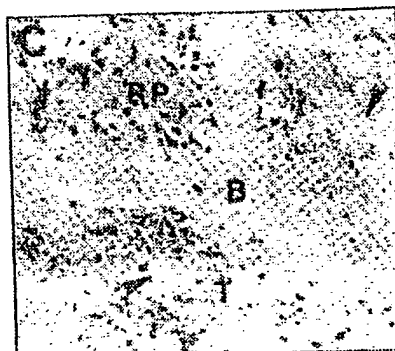
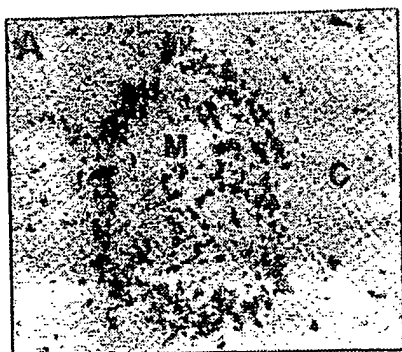
64. Spencer, S.C. and J.W. Fabre. 1990. Characterization of the tissue macrophage and the interstitial dendritic cell as distinct leukocytes normally resident in the connective tissue of rat heart. *J. Exp. Med.* 171:1841.
65. Tschachler, E., G. Schuler, J. Huttterer, H. Leibl, K. Wolff, and G. Stingl. 1983. Expression of Thy-1 antigen by murine epidermal cells. *J. Invest. Dermatol.* 81: 282.
66. Bergstresser, P.R., R.E. Tigelaar, J.H. Dees and J.W. Streilein. 1983. Thy-1 antigen-bearing dendritic cells populate murine epidermis. *J. Invest. Dermatol.* 81:286.
67. Lefrançois, L. 1991. Intraepithelial lymphocytes of the intestinal mucosa: curiouser and curiouser. *Semin. Immunol.* 3:99.
68. Rowden, G., M.G. Lewis, and A.K. Sullivan. 1977. Ia antigen expression on human epidermal Langerhans cells. *Nature (Lond.)*.
69. Schuler, G., and R.M. Steinman. 1985. Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J. Exp. Med.* 161:526.
70. Cumberbatch, M., and I. Kimber. 1990. Phenotypic characteristics of antigen-bearing cells in the draining lymph nodes of contact sensitized mice. *Immunology.* 71:404.
71. Larsen, C.P., R.M. Steinman, M. Witmer-Pack, D.F. Hankins, P.J. Morris, and J.M. Austyn. 1990. Migration and maturation of Langerhans cells in skin transplants and explants. *J. Exp. Med.* 172:1483

CLAIMS

1. A ligand which binds a binding partner which is able to bind with the antibody MRC OX-62 which is produced by the hybridoma deposited as ECACC 91061805.
- 5 2. A ligand according to claim 1 which is an antibody or an antibody fragment.
3. A mutant, derivative or functional equivalent of a
10 ligand according to claim 1 or claim 2, which mutant, derivative or functional equivalent binds the binding partner which is able to bind the antibody MRC OX-62 which is produced by the deposited hybridoma ECACC 91061805.
- 15 4. A derivative according to claim 3 which is a chimeric molecule.
5. A cell which produces a ligand according to claim 1
20 or claim 2.
6. A cell which produces a mutant, derivative or functional equivalent according to claim 3.
- 25 7. An antibody as obtainable from the hybridoma MRC OX-62 deposited as ECACC 91061805.

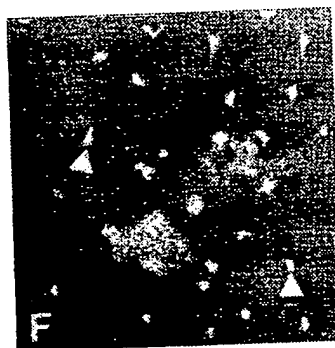
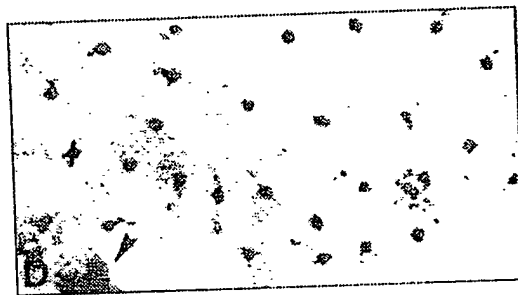
8. A mutant, derivative, functional equivalent or fragment of an antibody according to claim 7.
- 5 9. The hybridoma MRC OX-62, deposited as ECACC 91061805.
10. A mutant, derivative or descendant of the hybridoma of claim 9.
- 10 11. A binding partner able to bind with the antibody MRC OX-62 which is produced by the hybridoma deposited as ECACC 91061805.
- 15 12. A binding partner according to claim 11 which comprises all or part of a 150kD protein obtainable from dendritic cells.

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Fig.1.



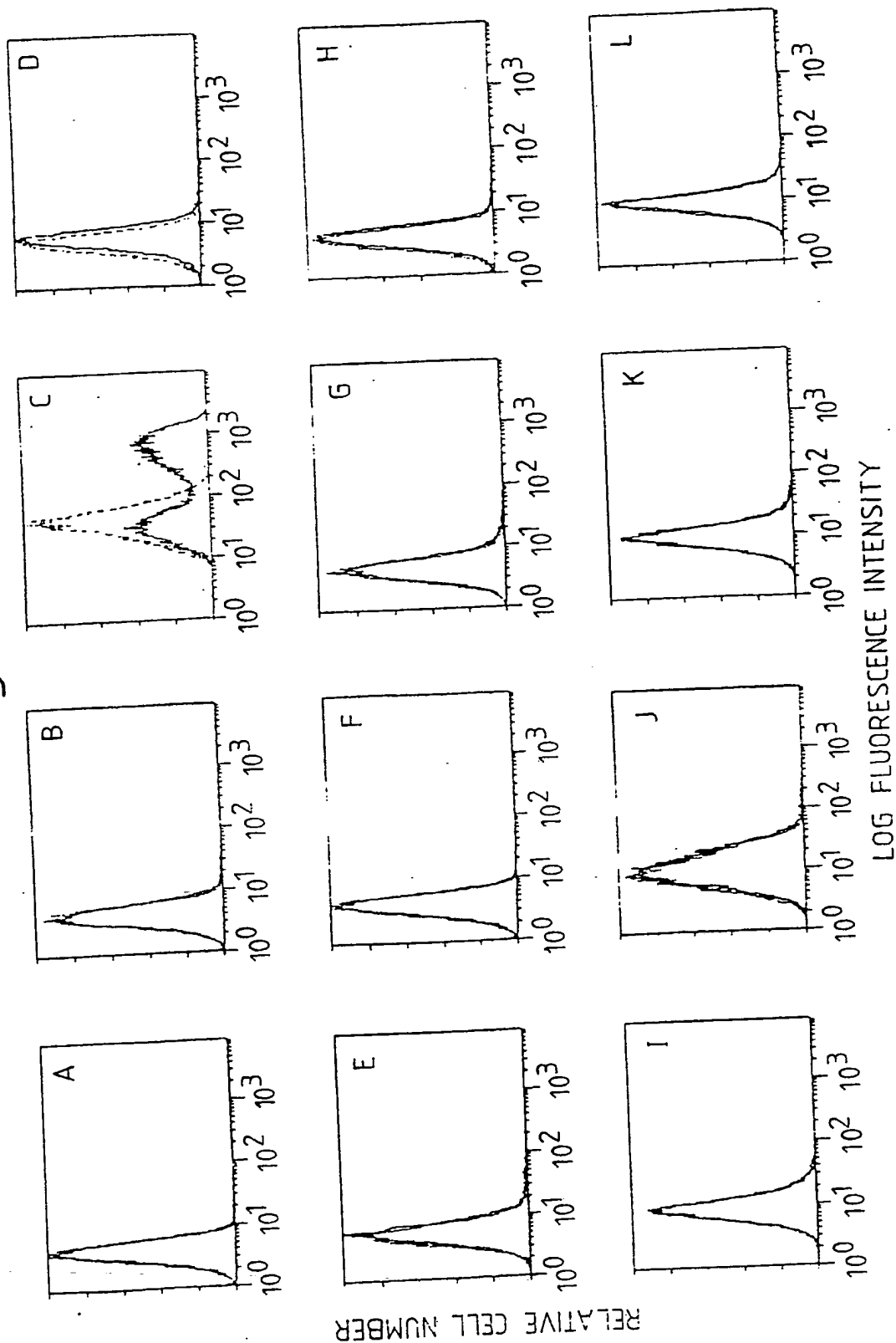
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Fig. 2.



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Fig. 3.



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Fig. 4.

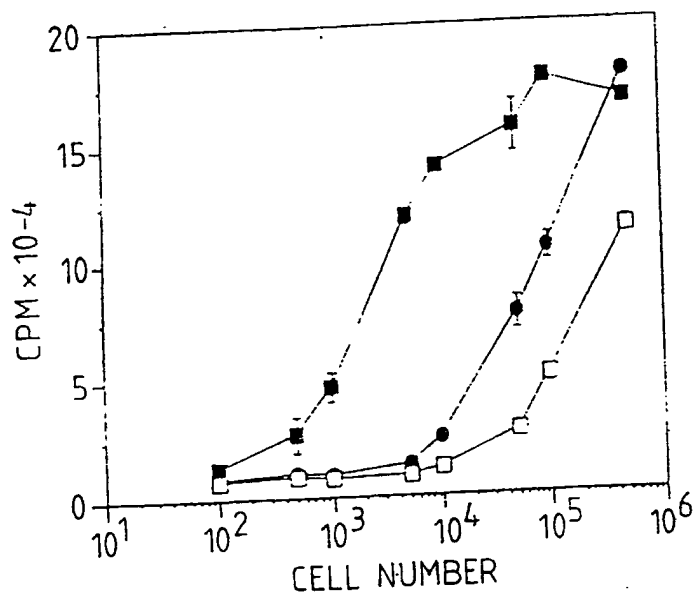
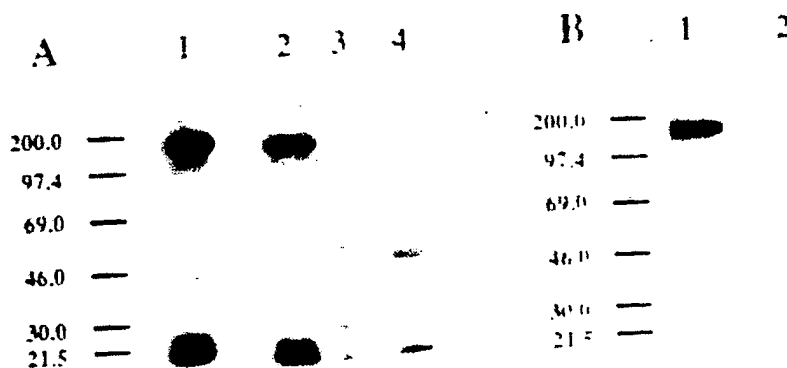


Fig. 5.



Applicant's or agent's file
reference number

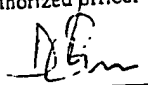
B/CP9503764

International application no.

PCT/GB92/01506

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>34</u> , line <u>4-10</u>	
Further deposits are identified on an additional sheet <input type="checkbox"/>	
B. IDENTIFICATION OF DEPOSIT	
Name of depositary institution European Collection of Animal Cell Cultures	
Address of depositary institution (including postal code and country) PHLS Centre for Applied Microbiology and Research Porton down Salisbury Wiltshire SP4 0JG GB	
Date of deposit 18 June 1991	Accession Number 91061805
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input checked="" type="checkbox"/>	
1. The applicants give notice that samples of the above deposited culture shall be available only to experts, in accordance with paragraph 3 of Schedule 2 to the Patents Rules 1990.	
2. The applicants give notice that until publication of the mention of the grant of a European patent or until the date on which the application is refused or	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
1. GB 2. EP 3. FI respectively	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
For receiving Office use only	
<input type="checkbox"/> This sheet was received with the international application	
Authorized officer	
For International Bureau use only	
<input checked="" type="checkbox"/> This sheet was received by the International Bureau on:	
29 OCT 1992 (29. 10. 92)	
Authorized officer	
	

6492/1566 29. 10. 92

C ADDITIONAL INDICATIONS (Continuation)

withdrawn or is deemed to be withdrawn, the above deposited culture shall be made available as provided in Rule 28(3) of the Implementing Regulations under the European Patent Convention only by the issue of a sample to an expert nominated by the requester, in accordance with Rule 28(4) of said Implementing Regulations.

3. The applicants request that, until the application has been laid open to public inspection by the National Board of Patents and Registration, or has been finally decided upon by the National Board of Patents and Registration without having been laid open to public inspection, the furnishing of a sample of the above deposited culture shall only be effected to an expert in the art.

29 OCT 1992 (29. 10. 92)

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INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/01506

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 12 P 21/08, C 07 K 15/00, A 61 K 39/395, G 01 N 33/569		
II. FIELDS SEARCHED		
Classification System	Minimum Documentation Searched ⁷	
IPC5	Classification Symbols A 61 K; C 07 K; C 12 P	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	Eur. J. Immunol., vol. 17, 1987, Matthijs Bree1 et al.: "Dendritic cells of the mouse recognized by two monoclonal antibodies ", pp. 1555-1559, see page 1559, left col. <div style="text-align: center;">--</div>	1-6,11, 12
A	J. Exp. Med., vol. 163, April 1986, Georg Kraal et al.: "Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody ", pp. 981-997, see page 990, fig 10, line B and Table I <div style="text-align: center;">--</div>	1-6,11, 12
A	Proc. Natl. Acad. Sci. USA, vol. 79, January 1982, M.C. Nussenzweig et al.: "A monoclonal antibody specific for mouse dendritic cells ", pp. 161-165 <div style="text-align: center;">--</div>	1-6,11, 12
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents:¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 28th October 1992		Date of Mailing of this International Search Report 13 NOV 1992
International Searching Authority EUROPEAN PATENT OFFICE		Signature of Authorized Officer Carl Olof Gustafsson

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	J. Exp. Med., vol. 171, May 1990, Joshua P. Metlay et al.: "The distinct leukocyte integrins of mouse spleen dendritic cells and identified with new hamster monoclonal antibodies", pp. 1753-1771, see pages 1757-60 and "Discussion"	1-6,11, 12
A	Dialog Information Services, Medline, file 154, 66-92/May, accession no. 07378483, Dupuy P. et al: "T-cell receptor-gamma/delta bearing lymphocytes in normal and inflammatory human skin", J Invest Dermatol Jun 1990, 94(6) p764-8	1-6,11, 12
X	National Library of Medicine database, Medline, file Med 89, NLM Accession no. 89279162, Maxfield S.R. et al: "Murine T cells express a cell surface receptor for multiple extracellular matrix proteins. Identification and characterization with monoclonal antibodies, & J. Exp. Med. 1989, June; 161(6) p2173-90	1-6,11, 12
X	Proc. Natl. Acad. Sci. USA, vol. 86, June 1989, Wendy L. Havran et al.: "Limited diversity of T-cell receptor gamma-chain expression of murine Thy-1+ dendritic epidermal cells revealed by Vgamma3-specific monoclonal antibody", see page 4185 - page 4189	1-6,11, 12
A	Res. Immunol., vol. 140, 1989, G. Kraal: "Immunocytochemistry of dendritic cells. A clue to their function?", see page 891 - page 895	1
A	National Library of Medicine database, Medline, File Med 91, NLM Accession no. 91010844, Crowley M.T. et al: "Use of the fluorescence activated cell sorter to enrich dendritic cells from mouse spleen",	1

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	<p>WO, A1, 9109620 (INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)) 11 July 1991, see the whole document</p> <p style="text-align: center;">-----</p>	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 92/01506

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This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 30/09/92
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9109620	11/07/91	FR-A- 2656532	05/07/91

For more details about this annex : see Official Journal of the European patent Office, No. 12/82